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Two new genes from the human ATP-binding cassette transporter superfamily, *ABCC11* and *ABCC12*, tandemly duplicated on chromosome 16q12

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Abstract

Several years ago, we initiated a long-term project of cloning new human ATP-binding cassette (ABC) transporters and linking them to various disease phenotypes. As one of the results of this project, we present two new members of the human ABCC subfamily, *ABCC11* and *ABCC12*. These two new human ABC transporters were fully characterized and mapped to the human chromosome 16q12. With the addition of these two genes, the complete human ABCC subfamily has 12 identified members (ABCC1–12), nine from the multidrug resistance-like subgroup, two from the sulfonylurea receptor subgroup, and the *CFTR* gene. Phylogenetic analysis determined that *ABCC11* and *ABCC12* are derived by duplication, and are most closely related to the *ABCC5* gene. Genetic variation in some ABCC subfamily members is associated with human inherited diseases, including cystic fibrosis (*CFTR/ABCC7*), Dubin–Johnson syndrome (*ABCC2*), pseudoxanthoma elasticum (*ABCC6*) and familial persistent hyperinsulinemic hypoglycemia of infancy (*ABCC8*). Since *ABCC11* and *ABCC12* were mapped to a region harboring gene(s) for paroxysmal kinesigenic choreoathetosis, the two genes represent positional candidates for this disorder.

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Abbreviations: ABC, ATP-binding cassette; BAC, bacterial artificial chromosome; bp, base pair(s); CEM, a human T-lymphoid cell line; CEM-r1, a PMEA-resistant variant of CEMss; CEMss, a human T-lymphoid cell line sensitive to PMEA; EST, expressed sequence tag; GSH, glutathione; ICCA, infantile convulsions with paroxysmal choreoathetosis; MRP, multidrug resistance protein; MTX, methotrexate; PCR, polymerase chain reaction; PKC, paroxysmal kinesigenic choreoathetosis; PMEA, 9-(2-phosphonylmethoxyethyl) adenine; 3TC, 2',3'-dideoxy-3'-thiacytidine; UTR, untranslated region

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1. Introduction

The ATP-binding cassette (ABC) transporter superfamily is one of the largest gene families and encodes a functionally diverse group of membrane proteins involved in energy-dependent transport of a wide variety of substrates across membranes (Dean and Allikmets, 1995). Phylogenetic analysis further divides human ABC transporters into seven subfamilies: ABCA (ABC1 subfamily), ABCB (MDR/TAP subfamily), ABCC (CFTR/MRP subfamily), ABCD (ALD subfamily), ABCE (OABP subfamily), ABCF (GCN20 subfamily), and ABCG (white subfamily)

(Allikmets et al., 1996; <http://www.gene.ucl.ac.uk/users/hester/abc.html>). Most ABC proteins from eukaryotes encode so-called 'full transporters', each consisting of two ATP-binding domains and two transmembrane domains (Hyde et al., 1990).

The human ABCC subfamily currently has ten identified members (ABCC1–10), seven from the multidrug resistance-like (MRP) subgroup, two from the sulfonylurea receptor (SUR) subgroup, and the *CFTR* gene. MRP-like proteins are organic anion transporters, i.e. they transport anionic drugs, exemplified by methotrexate (MTX), as well as neutral drugs conjugated to acidic ligands, such as glutathione (GSH), glucuronate, or sulfate, and play a role in resistance to nucleoside analogs (Cui et al., 1999; Kool et al., 1999; Schuetz et al., 1999; Wijnholds et al., 2000). Genetic variation in some ABCC subfamily members is associated with human inherited diseases, including cystic fibrosis (*CFTR/ABCC7*) (Riordan et al., 1989), Dubin–Johnson syndrome (*ABCC2*) (Wada et al., 1998), pseudoxanthoma elasticum (*ABCC6*) (Bergen et al., 2000; Le Saux et al., 2000) and familial persistent hyperinsulinemic hypoglycemia of infancy (*ABCC8*) (Thomas et al., 1995).

Paroxysmal kinesigenic choreoathetosis (PKC; MIM# 128200), the most frequent type of paroxysmal dyskinesia, is a disorder characterized by recurrent, frequent attacks of involuntary movements and postures, including chorea and dystonia, induced by sudden voluntary movements, stress, or excitement (for a detailed description of clinical and genetic features, see Swoboda et al., 2000). In most families it is inherited as an autosomal dominant trait with incomplete penetrance. The gene locus has been mapped to human chromosome 16q11–q12 (Tomita et al., 1999; Bennett et al., 2000).

We initiated a long-term project of cloning new human ABC transporters and linking them to various disease phenotypes (Allikmets et al., 1996, 1997, 1999). As one of the results of this project, we present here two new members of the human ABCC subfamily, *ABCC11* and *ABCC12*.

2. Materials and methods

2.1. Sequence analysis

Searches of the GenBank HTGS database were performed with the TBLASTN and TBLASTP programs on the NCBI file server (<http://www.ncbi.nlm.nih.gov>) with the known ABC transporter nucleotide and protein sequences as queries. Potential transmembrane spanning segments were predicted with the TMAP program (<http://bioweb.pasteur.fr/seqanal/interfaces/tmap.html>). Amino acid alignments were generated with the PILEUP program included in the Genetics Computer Group (GCG) Package. The GRAIL and GeneScan programs on Genome Analysis Pipe-

line I (<http://compbio.ornl.gov/GP/>) were utilized to predict genomic structures of the new genes.

2.2. cDNA cloning and determining the genomic structure

Primers were designed from expressed sequence tag (EST) clone sequences and from predicted cDNA sequences from 5' and 3' regions of genes. cDNA sequences of *ABCC11* and *ABCC12* were confirmed by PCR amplification of testis or liver cDNA (Clontech). Sequencing was performed on the ABI 377 sequencer according to the manufacturer's protocols (Perkin Elmer). Positions of introns were determined by comparison between genomic (BAC AC007600) and cDNA sequences. The sequence of the *ABCC11* and *ABCC12* cDNA was deposited with the GenBank Database under the accession numbers AY040219 and AY040220, respectively.

2.3. Physical mapping

The chromosomal localization of the human *ABCC11* and *ABCC12* genes was determined by mapping on the GeneBridge4 radiation hybrid panel (Research Genetics), according to the manufacturer's protocol.

2.4. Expression analysis

Expression profiles of the human *ABCC11* and *ABCC12* genes were determined by PCR on human Multiple Tissue cDNA (MTC™, Clontech) Panels I and II according to the manufacturer's instructions. Each MTC panel contains normalized, first-strand cDNA from eight human tissues/cells: (I) heart, whole brain, placenta, lung, liver skeletal muscle, kidney and pancreas; (II) spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocyte. The following primer pairs amplified specific gene products: *ABCC11*: forward 5'-AGA ATG GCT GTG AAG GCT CAG CAT C-3', reverse 5'-GTT CCT CTC CAG CTC CAG TGC-3'; *ABCC12*: forward 5'-GGT GAC AGA CAA GCG AGT TCA GAC AAT G-3', reverse 5'-CTT TGC TCC TCT GGG CCA GTG-3'.

2.5. Cell lines

The human erythroleukemia K562 cells were obtained from the American Tissue Culture Collection (Rockville, MD) and were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. The 9-(2-phosphonylmethoxyethyl)adenine (PMEA) resistant cells, K562/PMEA, were derived as described earlier (Hatse et al., 1996), and were kindly provided by Dr Jan Balzarini (Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium). The T-lymphoblast cell lines CEM and (-)2',3'-dideoxy-3'-thiacytidine (3TC)-resistant CEM-3TC cells were provided by Dr Guido Antonelli (Department of Experimental Medicine and Pathology Virology Section, University "La Sapienza", Rome, Italy). The selection of the 3TC-resistant cell line and its pheno-

Table 1
Splice site sequences and exon sizes for *ABCC11* and *ABCC12^a*

Exon and intron sequences are shown in uppercase and lowercase, respectively.

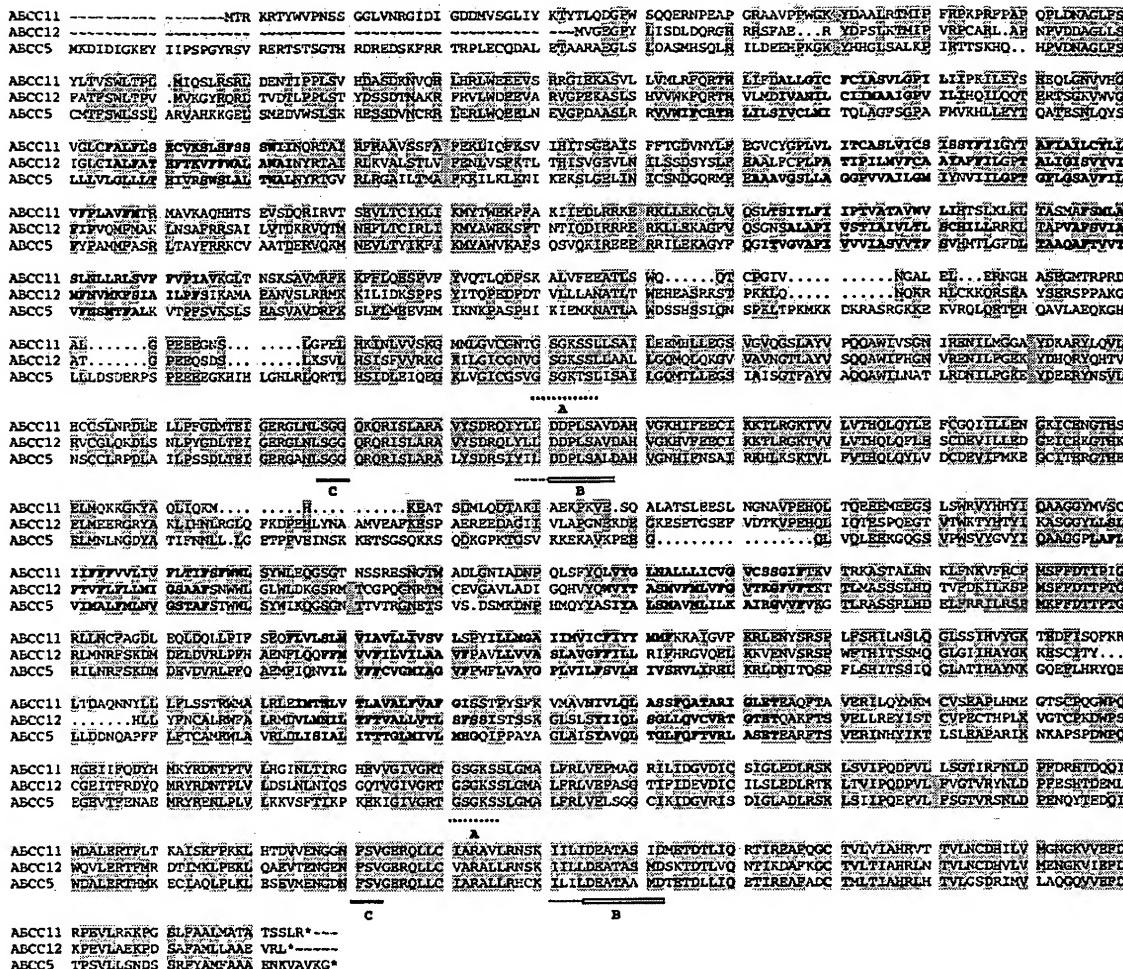


Fig. 1. Amino acid alignment of ABCC11, ABCC12, and ABCC5 proteins. Identical amino acids are shaded and gaps are indicated by periods. Walker A and B motifs and the ABC transporter family signature sequence C are underlined and labeled with respective letters. Potential transmembrane spanning segments are given in bold type.

typic properties will be described in detail in an upcoming publication. Another previously described pair of cell lines, CEMss and CEM-r1, were acquired from Dr Arnold Fridland (Robbins et al., 1995). CEM-r1 is highly resistant to PMEA due to an overexpression of *ABCC4* (Schuetz et al., 1999). Total RNA from these six cell lines (three pairs of wild-type and resistant cell lines) was isolated with TRIZOL (GIBCO BRL), and RT-PCR was performed at varying

cycle numbers with primers described in Section 2.4. The PCR products were subcloned into the pCR 2.1 vector and verified by direct sequencing.

2.6. Phylogenetic analysis

Complete protein sequences were aligned with the CLUSTALW program (Thompson et al., 1994). The result-

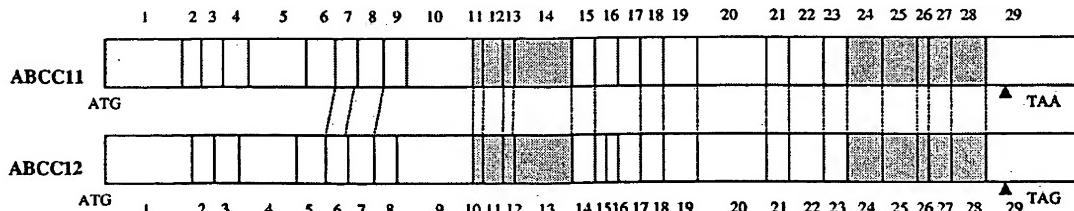


Fig. 2. Splicing pattern comparison of the ABCC11 and ABCC12 genes. Clear boxes represent exons and vertical lines define splice sites. Exon numbers for each gene are shown both above and below the drawing. Filled boxes indicate the exons encoding ABC domains. Regions in which the two genes show identical splicing patterns are indicated by dashed lines.

ing multiple alignment was analyzed with program NJBOOT by N. Takezaki (pers. commun.) implementing the neighbor-joining tree-making algorithm (Saitou and Nei, 1987). The Poisson correction for multiple hits (Zuckerkandl and Pauling, 1965) was used as the distance measure between sequences for generating a phylogenetic tree.

3. Results and discussion

3.1. Cloning and genomic structure of ABCC11 and ABCC12

Two new human ABC transporter gene sequences were detected on the bacterial artificial chromosome (BAC) clone #AC007600 from the GenBank HTGS database. cDNA sequencing, genomic structure prediction programs, and computer searches determined the sequence and genomic structure of both new genes belonging to the ABCC (MRP) subfamily. Only the combination of all these methods allowed for the correct assembly of these genes which are closely related and highly conserved in evolution.

The human ABCC11 and ABCC12 genes consist of 29 exons. Exon sizes range from 72 to 252 bp for ABCC11 and from 73 to 279 bp for ABCC12. All exons were flanked by GT and AG dinucleotides consistent with the consensus sequences for splice junctions in eukaryotic genes (Table 1). Of the 28 introns in ABCC11, 18 are class 0 (where the splice occurs between codons), four are class 1 (where the codon is interrupted between the first and the second nucleotide), and six are class 2 (where the splice occurs between the second and the third nucleotide of the codon). For the ABCC12 gene these numbers are 16, six and six, respectively. The ABCC11 gene encodes a protein of 1382 amino acids, and ABCC12 a protein of 1359 amino acids (Fig. 1). Topology predictions based on hydropathy profiles and comparison with other known ABC transporters suggest that both encoded proteins are full ABC transporters containing two ATP-binding domains (including Walker A and B domains, and signature motifs) and two transmembrane domains (Fig. 1). The amino acid sequence of ABCC11 is 40% identical to the human ABCC5 protein, 33% identical to human ABCC4 and 32% identical to ABCC2 and ABCC3 proteins. The ABCC12 protein is even more closely related to ABCC5 (42% identity on protein level; Fig. 1).

The splicing pattern of the two new genes is very similar, especially towards the 3' end (Fig. 2), suggesting a close evolutionary relationship between these ABC transporters. The ABCC11 and ABCC12 proteins, as well as ABCC4 and ABCC5, are smaller than another well-known member of the subgroup, ABCC1 (MRP1), appearing to lack the extra N-terminal domain (Fig. 1) (Borst et al., 2000). It has been shown, however, that the extra N-terminal part of ABCC1 is not required for the transport function (Bakos et al., 1998).

The ABCC4 and ABCC5 proteins confer resistance to nucleotide analogs, including PMEA and purine base analogs (Schuetz et al., 1999; Wijnholds et al., 2000). ABCC1, ABCC2 and ABCC3 transport drugs conjugated to GSH, glucuronate, sulfate and other organic anions, such as MTX (Cui et al., 1999; Kool et al., 1999; Wijnholds et al., 2000). Since structurally related ABC proteins often transport similar substrates across cell membranes, it would be reasonable to suggest that ABCC11 and ABCC12 could share functional similarities with ABCC4 and/or ABCC5.

3.2. Expression of ABCC11 and ABCC12 in human tissues and nucleoside-resistant cell lines

The expression patterns for the ABCC11 and ABCC12 genes were examined by PCR on MTC panels (Clontech) with gene-specific primers resulting in about 500 bp PCR fragments (Fig. 3B). ABCC11 was expressed in all tissues except kidney, spleen, and colon. The ABCC12 transcript was detected, at much lower levels, only in testes, ovary, and prostate. The size for both transcripts was determined at approximately 5000 bp on MTN blots (Clontech, data not shown). The primers used in expression studies amplified the ABCC11 cDNA from exon 7 to exon 10, resulting in a 527 bp PCR fragment (Fig. 3B). In the case of lung, and occasionally some other tissues (data not shown), a smaller (419 bp) fragment was detected (Fig. 3B). Direct sequen-

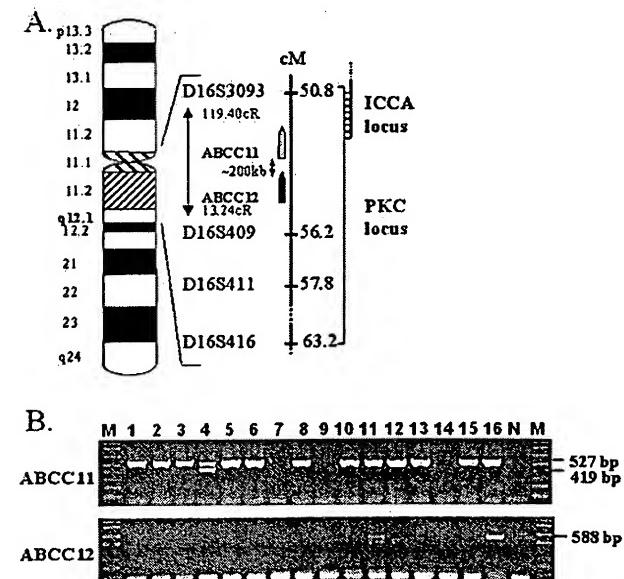


Fig. 3. Chromosomal localization and expression analysis of the ABCC11 and ABCC12 genes. (A) Human ABCC11 and ABCC12 genes, flanked by markers D16S3093 and D16S409, are separated by ~200 kb, and organized in a head-to-tail fashion, with their 5' ends facing the centromere. Loci for ICCA, PKC, and their overlap, are defined by brackets. (B) Expression analysis of the human ABCC11 and ABCC12 genes by PCR on MTC panels. Lanes 1–16 represent cDNA from heart, brain, placenta, lung, liver, muscle, kidney, pancreas, spleen, thymus, testis, ovary, intestine, colon, leukocyte, and prostate, respectively. N, negative control; M, marker lane (1 kb Plus DNA Ladder).

cing of the PCR product determined that the shorter PCR product lacked exon 9 of the *ABCC11* gene. Since these results were confirmed in repeated experiments, frequent skipping of *ABCC11* exon 9 may occur *in vivo*. Exon skipping and alternative splicing events have been well-documented for several ABC genes (Rickers et al., 1994; Bellincampi et al., 2001).

Systematic analysis of the tissue source of the *ABCC12* ESTs from the public dbEST and the proprietary Incyte LifeSeq Gold databases indicates that 11/18 of the matching sequences are derived from various CNS origins, and the rest are from testis (three clones) and immune system (four clones). Similar analysis for the *ABCC11* gene resulted in 29 ESTs, with the majority being derived from breast tumor tissue (17). The others were from prostate (five clones), testis (three), CNS (two), and colon (two). Certain discrepancies between the two expression profiling methods are often observed for low abundance transcripts, which have high tissue distribution selectivity.

Since the new genes show extensive structural similarity to ABCC5 (and to a certain extent, ABCC4), we checked their expression in three pairs of cell lines, K562 and K562-PMEA, CEMss and CEM-r1, and CEM and CEM-3TC. The K562-PMEA and CEM-r1 lines have been selected for resistance to PMEA, and the CEM-3TC for resistance to

the cytidine nucleoside analog, 3TC. No difference was observed in expression levels of *ABCC11* between the parental and PMEA-resistant cell lines. In contrast, the CEM-3TC cell line revealed a reproducible two- to three-fold increase in the expression of *ABCC11*, when compared to the parental line CEM (data not shown). This is a potentially interesting finding when one considers the close evolutionary relationship of *ABCC11* and *ABCC5* (Figs. 1 and 4), and that a recent study by Borst and colleagues (Wijnholds et al., 2000) has demonstrated selective nucleotide analog transport by *ABCC5*. In addition, since the efflux-resistant phenotype of CEM-3TC can be explained only in part by *ABCC4* overexpression (J.D.S., unpublished data), the higher expression of *ABCC11* in these cells warrants further investigation.

3.3. Radiation hybrid mapping

Radiation hybrid mapping placed *ABCC11* and *ABCC12* to the centromeric region of human chromosome 16, flanked by markers D16S3093 and D16S409 (Fig. 3A). The region encompasses 5.4 cM, or 132.5 cR, and could not be narrowed down further due to lack of recombination and/or mapped polymorphic markers in this region. Both genes are most likely localized on chromosome 16q12.1, since

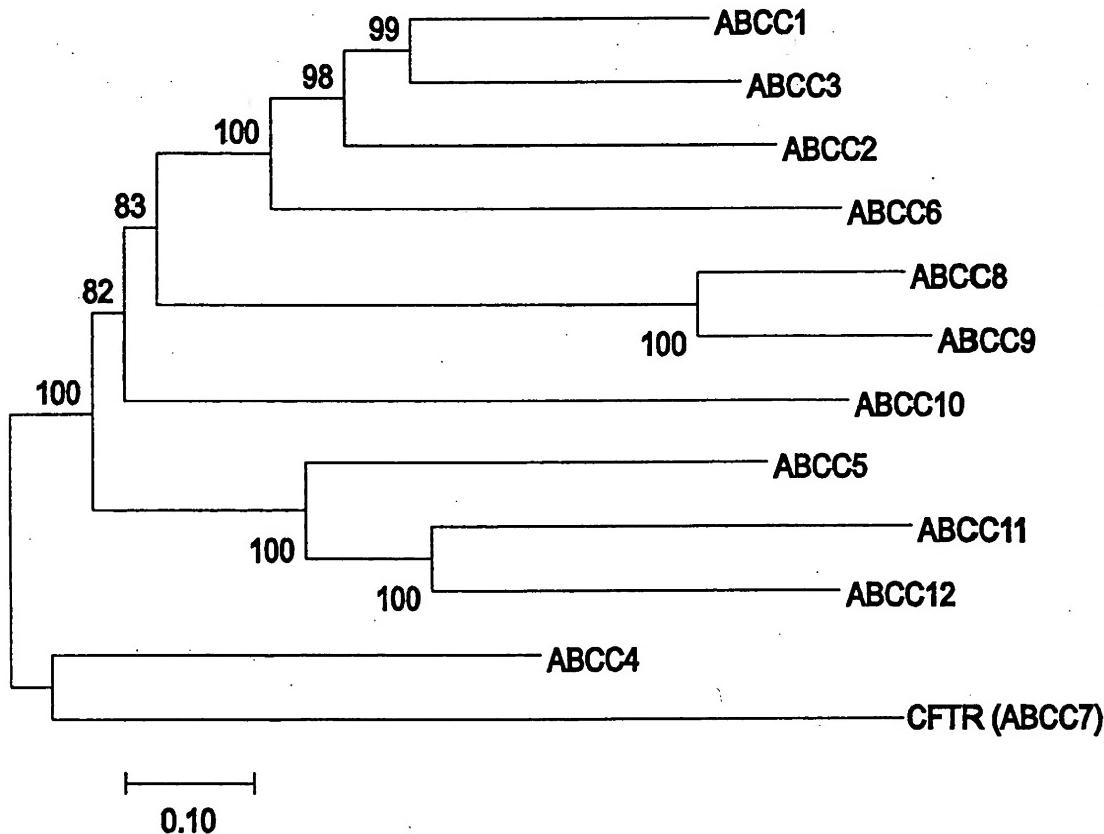


Fig. 4. Phylogenetic relationship of genes in the ABCC subfamily. Complete protein sequences of all members of the ABCC subfamily were aligned with the CLUSTALW program. The distance measure is given in substitutions per amino acid.

they map much closer to the 16q marker D16S409 (13.24 cR) than the 16p marker D16S3093 (119.40 cR) (Fig. 3A). The location of the new genes was confirmed also by LANL BAC mapping data, where the BAC clone #AC007600 was mapped to 16q12.1 by the STS marker s9B1 (http://www.jgi.doe.gov/JGI_home.html). *ABCC11* and *ABCC12* are located tandemly, separated by about 200 kb, with their 5' ends facing towards the centromere (Fig. 3). Two more ABCC subfamily genes, *ABCC1* and *ABCC6*, have been mapped to the short arm of the same chromosome, at 16p13.1 (Cole et al., 1992; Allikmets et al., 1996). The 3' ends of *ABCC1* and *ABCC6* are only about 9 kb apart from each other so the genes face opposite directions (Cai et al., 2000).

3.4. Phylogenetic analyses

Phylogenetic analyses of the ABCC subfamily proteins clearly demonstrate a relatively recent duplication of the *ABCC11* and *ABCC12* genes (Fig. 4). The resulting neighbor-joining tree shows with maximum confidence (100% level of bootstrap support) a close evolutionary relationship of the *ABCC11/ABCC12* cluster with the *ABCC5* gene (Fig. 4). In addition, the analysis of the tree suggests a recent duplication of the *ABCC8* and *ABCC9* genes, while *ABCC10* seems to be one of the first genes to separate from the common ancestor. *ABCC1*, *ABCC2*, *ABCC3*, and *ABCC6* genes constitute a well-defined sub-cluster, while the *ABCC4* and *CFTR* (*ABCC7*) genes form another reliable subset despite apparent early divergence.

3.5. *ABCC11* and *ABCC12* as candidate genes for PKC

The locus for PKC has been assigned to 16p11.2-q12.1, between markers D16S3093 and D16S416 (Tomita et al., 1999; Bennett et al., 2000) (Fig. 3A). An overlapping locus has been predicted to contain the gene for infantile convulsions with paroxysmal choreoathetosis (ICCA; Lee et al., 1998). Expression analysis by PCR and by EST database mining suggests that the two genes are expressed in tissues (CNS, muscle) potentially involved in the etiology of PKC. In summary, chromosomal localization, potential function, and expression profiles make both genes promising candidates for PKC/ICCA. Preliminary analysis of the *ABCC11* gene has identified several single nucleotide polymorphisms, including an amino acid-changing variant (S6G > A, R19H) in the first exon. Complete screening of the *ABCC11* and *ABCC12* genes for genetic variation in families segregating PKC is currently under way.

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